Microbial Interaction in a Symbiotic Bioprocess of Lactic Acid Bacterium and Diary Yeast

Hiroshi Shimizu[1], Sunao Egawa[2], Agustin K. Wardani[2], Keisuke Nagahisa[1], and Suteaki Shioya[2]

[1] Dept. of Bioinformatic Engineering, Graduate School of Information Science and Technology, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
{shimizu, nagahisa}@ist.osaka-u.ac.jp
http://www-shimizu.ist.osaka-u.ac.jp/mej.html
[2] Dept. of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka, 565-0871, Japan
{agustin, shioya}@bio.eng.osaka-u.ac.jp

Abstract. In symbiotic processes, different organisms coexist stably and interact by sharing with same metabolites and environmental conditions. A symbiotic process of a lactic acid bacterium, *Lactococcus lactis* sub species *lactis* (ATCC11454) and diary yeast *Kluyveromyces marxianus* is studied in this paper. A mathematical model of the symbiotic process composed of two microorganisms is developed by integrating two pure cultivation models. A cascade pH controller coupled with the dissolved oxygen (DO) control is newly developed and lactate consumption activity of *K. marxianus* is controlled by changing the DO concentration. The pH and lactate are kept stably at constant levels and both microorganisms grow well. Stability of this symbiotic process with disturbance of inoculums sizes of both microorganisms is investigated. The dynamic behavior of fusion process of independent two bionetworks is also discussed.

1 Symbiotic Process and Microbial Interaction

Microbial ecosystem, which consists of abundant genus of microbial populations, takes an important role for maintaining a microcosm as well as carbon and nitrogen circulation in global environment. From ancient age, people utilize complex microbial functions to produce many substances, such as foods, brewing drinks, pharmaceuticals and so on. Most fermented foods are produced by mixed cultures acting on various substrates. Cheese, yogurt, pickles, whiskey and Japanese rice wine sake are some examples of fermented foods. Numerous interactions, such as competition, predation, commensalism, mutualism, happen between microbial communities. Especially, combination of lactic acid bacteria (LAB) and diary yeasts (DY) is the most popular for making diary and brewing products in the world. And their interaction, which is usually mutualism, affects taste

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and flavors of the product, stability and productivity in their processes. One of typical examples of microbial interaction of LAB and DY is studied in this paper.

Living organisms are complex systems with multidimensional hierarchical networks, composed of gene, protein, and metabolic networks, respectively. Living cells have ability to flexibly change the topology of complex bionetworks in order to survive under many unexpected environmental conditions. In symbiotic systems, bionetworks composed of two different microorganisms fuse together by sharing with same nutrients in the environments. In the case that nutrients are competitively taken by two organisms, competition phenomenon happens among two microorganisms. When metabolic wastes from one microorganism become nutrients for the other microorganism, commensalism phenomenon happens. When the metabolic wastes show inhibitory effect for the producing microorganisms, the cleaning up of the wastes by the different microorganism makes favorable condition and mutualism phenomenon happens [1]. In this paper, we study behavior of a symbiotic process with a lactic acid bacterium, *Lactococcus lactis*, and diary yeast, *Kluyveromyces marxianus*.

Certain strains of *L. lactis* produces a food preservative nisin[2]. In the LAB fermentation process, the growth inhibition happens due to the accumulation of lactate and the decrease in pH [3]. In this study, a new pH control strategy with microbial interaction was developed. The concept of this strategy is shown in Fig.1. The *L. lactis* assimilates maltose as a carbon source and produces lactate. *K. marxianus*, which was isolated from kefir grains, does not have ability to assimilate maltose, while it has ability to assimilate lactate. Since the consumption rate of lactate is affected by dissolved oxygen (DO) concentration, lactate concentration and pH are controlled by manipulation of DO concentration. One measure of symbiotic process is how good growth of both microorganisms is. Since nisin is produced as growth associated, nisin production is a good indicator of how good symbiotic process is working.

The activity of the microorganisms can be represented as specific reaction rates, that is, reaction rates per unit cell concentration. Specific reaction rates of both microorganisms, including specific growth rate and the specific production rate of lactate by *L. lactis*, and the specific consumption rate of lactate by *K. marxianus* are examined in pure cultures. Based on the information of

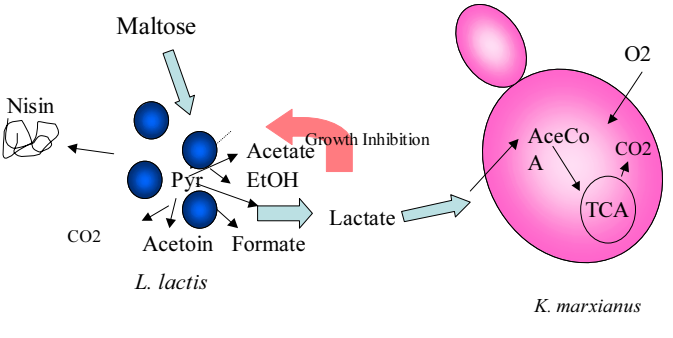

Fig. 1. Microbial interaction and removal of lactate

specific reaction rates of both microorganisms, a symbiotic process with microbial interaction of both microorganisms is developed. Nisin production is used as an indicator of the bioprocess and it is compared with that of pure cultivation process of *L. lactis*.

2 Materials and Methods

2.1 Microrganisms and Methods

L. lactis subsp. *lactis* ATCC 11454 was used as a nisin producing lactic acid bacterium. *K. marxianus* MS1 was isolated from kefir grains by ourselves. Concentrations of maltose, yeast extract, and peptone in main culture were 40, 10, and 10 g/L respectively, and for fermentation with high cell concentration they are set 60, 40, and 40 g/L, respectively.

2.2 Analysis

Cell concentrations of the pure cultivation processes were measured as dry cell mass and optical density (OD). The viable cell concentrations of *L. lactis* and *K. marxianus* in the symbiotic process were determined as colony forming units (CFU) on selection media. Concentrations of L-lactate, acetate, and formate in the medium were analyzed enzymatically. Ethanol concentration was measured by gas chromatography. Glucose concentration was measured using a glucose analyzer (Model 2700, YSI Inc., OH). Maltose concentration was measured after hydrolysis to glucose. Nisin concentration was measured by a bioassay method based on the method of Matsuzaki et al. [4].

2.3 Cultivation Method

Before main cultivation was performed, culture size was scaled up by two steps in order to increase the amount of cells with high growth activity. Main cultures were performed in a 5 L jar fermentor (EPC Control Box, Eyla, Japan) equipped with temperature, pH, dissolved oxygen (DO) concentration and gas flow control systems, respectively. The working volume was 2 L. Air or nitrogen was supplied to the fermentor for aerobic or anaerobic cultivation conditions, respectively. In this study, the cascade control strategy was applied in order to control pH level via DO control by manipulating the agitation speed. Other detailed methods were described previously [5].

3 Pure Cultivation Porcess of *L. lactis*

The time course of pure cultivation process of *L. lactis* under anaerobic conditions without pH control is as shown in Fig. 2. The pH decreased below 5.0 within 3 h due to increase in the produced lactate. The cell growth was completely terminated after 6 h and the concentration of nisin was 7.4 mg/L. The specific growth rate (μ_L) of *L. lactis* and specific production rate of nisin (ρ_N) without pH control were 0.30 h^{-1} and 4.0 mg-nisin/g-cell/h, respectively.

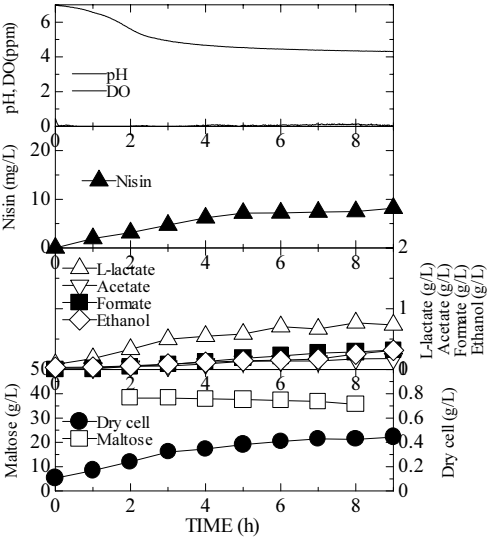

Fig. 2. Nisin production without pH control

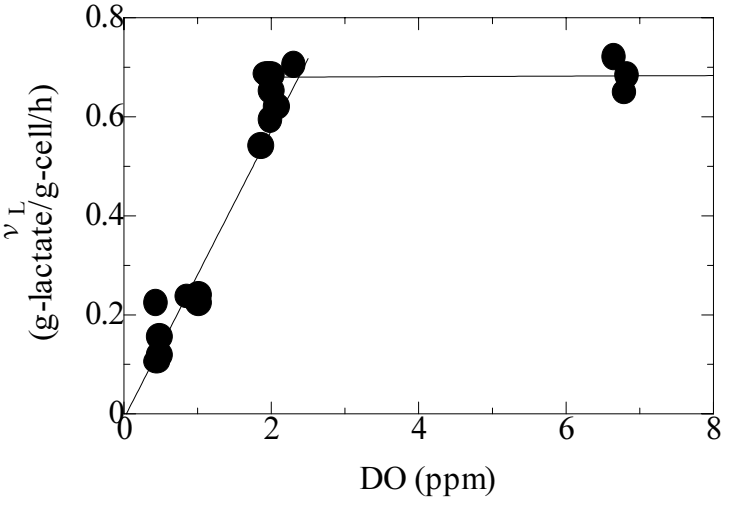

Fig. 3. Effect of DO concentration on the specific rate of consumption of lactate by *K. marxianus*

4 Pure Cultivation Process of *K. marxianus*

The specific lactate consumption rate of *K. marxianus* was determined under aerobic conditions. The effect of dissolved oxygen (DO) concentration on lactate consumption rate by *K. marxianus* is shown in Fig. 3. The maximum specific

rate of lactate consumption of *K. marxianus* (ν_L), was about 0.7 g/g-cell/h, which was greater than the maximum specific rate of lactate production of *L. lactis* under aerobic conditions. Thus, it was expected that lactate produced by *L. lactis* would be completely consumed by *K. marxianus*. The ν_L decreased linearly as DO concentration decreased in the range below 2 mg/L as shown in Fig. 3. When the lactate concentration was expected to be decreased, the DO concentration level should be increased and the specific rate of lactate consumption of *K. marxianus* is enhanced. On the other hand, lactate concentration was expected to be increased; the DO level should be decreased and the specific rate of lactate consumption of *K. marxianus* is attenuated.

5 Development of Symbiotic Bioprocess of *L. lactis* and *K. marxianus*

As shown in Fig.1, a symbiotic bioprocess of *L. lactis* and *K. marxianus* was developed. In order to keep lactate and pH at constant, a novel cascade control system was designed, taking into account of microbial interaction. Figure 4 shows a flow diagram of the automatic cascade controller of coupling of pH with DO control in the symbiotic process. The PI and PID control strategies were employed for the automatic control of DO and pH controllers.

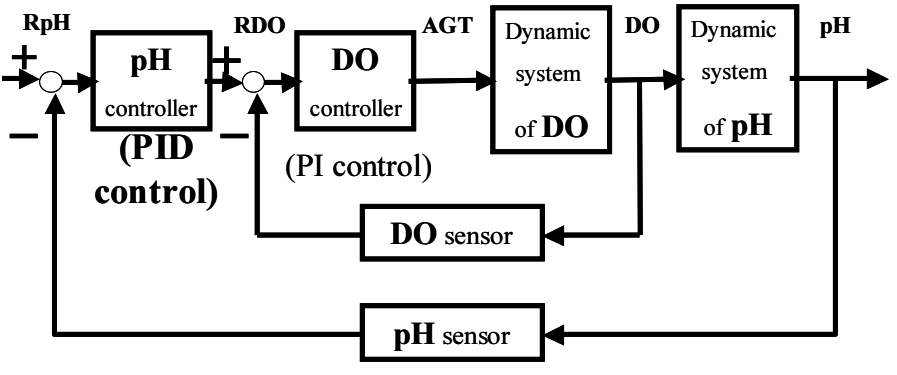

Fig. 4. A cascade pH controller incorporated with DO control

6 Development of Mathematical Model of Symbiotic Process of *L. lactis* and *K. marxianus*

A mathematical model of a symbiotic process was developed to optimize a pH cascade controller in the symbiotic process consisting *L. lactis* and *K. marxianus*. The symbiotic process model was developed by integrating individual models of *L. lactis* and *K. marxianus* in pure cultivation processes.

6.1 Mathematical Model of *L. lactis*

The Lactic acid bacterium *L. lactis* produce many metabolites hetero-fermenta tively such as lactate, acetate, acetoin, formate, nisin and so on. The concentrations of cell of *L.lactis* (X_L), maltose (S_M), lactate (L), acetate (A), acetoin (AT), formate (F), and nisin (N) are represented, respectively, as follows

Cell growth

$$\frac{dX_L}{dt} = \mu X_L \tag{1}$$

Maltose consumption

$$\frac{dS_M}{dt} = \nu_M X_L \tag{2}$$

Lactate production

$$\frac{dL}{dt} = \rho_L X_L \tag{3}$$

Acetate production

$$\frac{dA}{dt} = \rho_A X_L \tag{4}$$

Acetoin production

$$\frac{dAT}{dt} = \rho_{AT} X_L \tag{5}$$

Formate production

$$\frac{dF}{dt} = \rho_F X_L \tag{6}$$

Nisin production

$$\frac{dN}{dt} = \rho_N X_L \tag{7}$$

where μ_L, ν_M, ρ_L, ρ_A, ρ_{AT}, ρ_F, ρ_N are specific rates of growth, consumption of maltose, production of lactate, production of acetate, production of acetoin, production of formate, production of nisin, respectively. Effects of environmental conditions on specific rates are involved into the model mathematically [6].

Computer simulation was performed for the pure cultivation process of *L. lactis*, using the mathematical model. A satisfactory approximation to the experimental data was given by the mathematical model as shown in Fig. 5. Additionally, oxygen consumption was observed under aerobic condition. Oxygen consumption rate (q_{O2XL}) of *L. lactis* is calculated based on NADH/NAD^+ balance from the stoichiometric equations in the metabolic pathway as:

$$q_{O2XL} = \frac{\frac{4\nu_M}{MW_M} + \frac{\rho_A}{MW_A} + \frac{\rho_F}{MW_F}}{2} \tag{8}$$

where MW_M, MW_A, MW_F are molecular weights of maltose, acetate and formate, respectively.

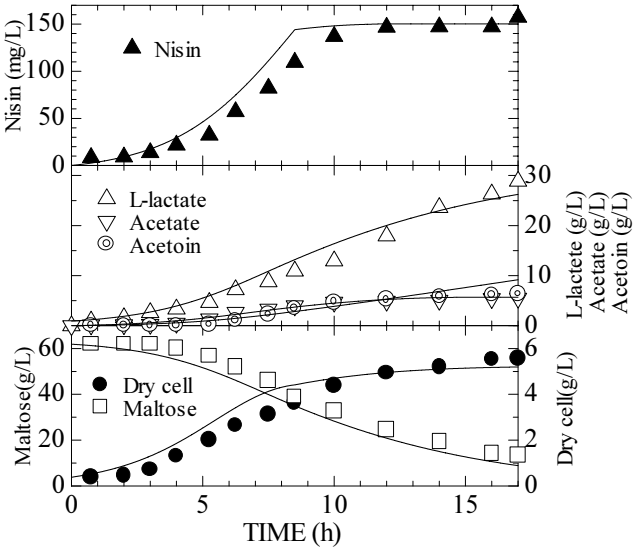

Fig. 5. Simulation and experimental result of pure cultivation process of *L. lactis*

6.2 Mathematical Model of *K. marxianus*

The cell growth and lactate consumption of *K. marxianus* are represented in Eqs. (9) and (10), respectively. Concentrations of cell of *K. marxianus*(X_K) and lactate (L) are represented as follows:

Cell growth

$$\frac{dX_K}{dt} = \mu_K X_K \tag{9}$$

Lactate

$$\frac{dL}{dt} = -\nu_L X_K \tag{10}$$

where μ_K and ν_L are specific rate of cell growth and lactate consumption of *K.marxinaus*, respectively. Effects of DO concentration on specific consumption rate of lactate is shown as in Fig. 3. Computer simulation for the pure cultivation process of *K. marxianus* was performed. It was found that the model gave a satisfactory approximation to the experimental data as shown in Fig. 6.

6.3 Mathematical Model of Symbiotic Process of *L.lactis* and *K. marxianus*

In the symbiotic process, both models of pure cultivation processes of *L. lactis* and *K. marxianus* were integrated into one model. Mass balance of lactate is represented as

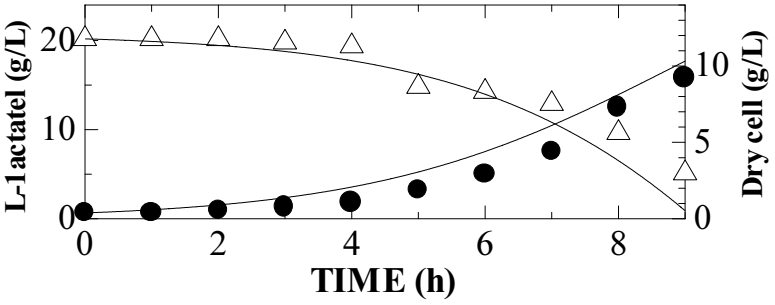

Fig. 6. Simulation and experimental result of pure cultivation process of *K. marxianus*

$$\frac{dL}{dt} = \rho_L X_L - \nu_L X_K \tag{11}$$

Since the dissolved oxygen (DO) concentration affected the specific consumption rate of *K. marxianus* as shown in Fig.3, balance of the DO concentration in the symbiotic process was involved in the model as:

$$\frac{dC}{dt} = k_L a(C^* - C) - M_{O2}(q_{O2XL} + q_{O2XK}) \tag{12}$$

where $k_L a$, C, C^*, are the mass transfer coefficient of oxygen, dissolved oxygen concentration and its saturated value, respectively. M_{O2}, q_{O2XL}, q_{O2XK} are the molecular weight of oxygen (defined as 32000 mg-O_2/mol), oxygen consumption of *L. lactis* and oxygen consumption of *K. marxianus*, respectively. The DO concentration was monitored by a DO sensor with delay shown as:

$$\frac{dC_{MES}}{dt} = k_{late}(C - C_{MES}) \tag{13}$$

where C_{MES} is the measured value of oxygen and k_{late} is a time constant, determined experimentally as 1/8 (1/sec). The dynamics of the pH change in the medium with time is described as shown in Eq. (14) as :

$$\frac{dpH}{dt} = \frac{-(\frac{\rho_L X_L - \nu_L X_L}{MW_L(1+10^{-pH+pK_L})} + \frac{\rho_A X_L}{MW_L(1+10^{-pH+pK_A})} + \frac{\rho_F X_L}{MW_F(1+10^{-pH+pK_F})})}{K + ln10(10^{-pH} + 10^{pH-14} + term_L + term_A + term_F)} \tag{14}$$

where $term_L$, $term_A$, and $term_F$ are represented, respectively as

$$term_L = \frac{L}{MW_L}\frac{10^{-pH+pK_L}}{(1+10^{-pH+pK_L})^2} \tag{15}$$

$$term_A = \frac{A}{MW_A}\frac{10^{-pH+pK_A}}{(1+10^{-pH+pK_A})^2} \tag{16}$$

$$term_F = \frac{F}{MW_F} \frac{10^{-pH+pK_F}}{(1+10^{-pH+pK_F})^2} \tag{17}$$

MW_L, pK_L, pK_A, pK_F, and K are the molecular weight of lactate, dissociation constants for lactic acid, acetate and formate, and a consatnt parameter, respectively.

7 Optimization of Symbiotic Control Process of *L. lactis* and *K. marxianus* by Simulation Study

To keep the lactate in low level by microbial interaction of *L. lactis* and *K. marxianus*, the cascade pH control system as shown in Fig. 4 was developed. Because the pH in the medium was controlled by the lactate consumption of *K. marxianus* and the specific lactate consumption rate was controlled by manipulating the DO concentration, a cascade controller coupled with the DO control was developed. The consumption rate of lactate by *K. marxianus* was decreased linearly as the DO concentration decreased in the range below 2 ppm

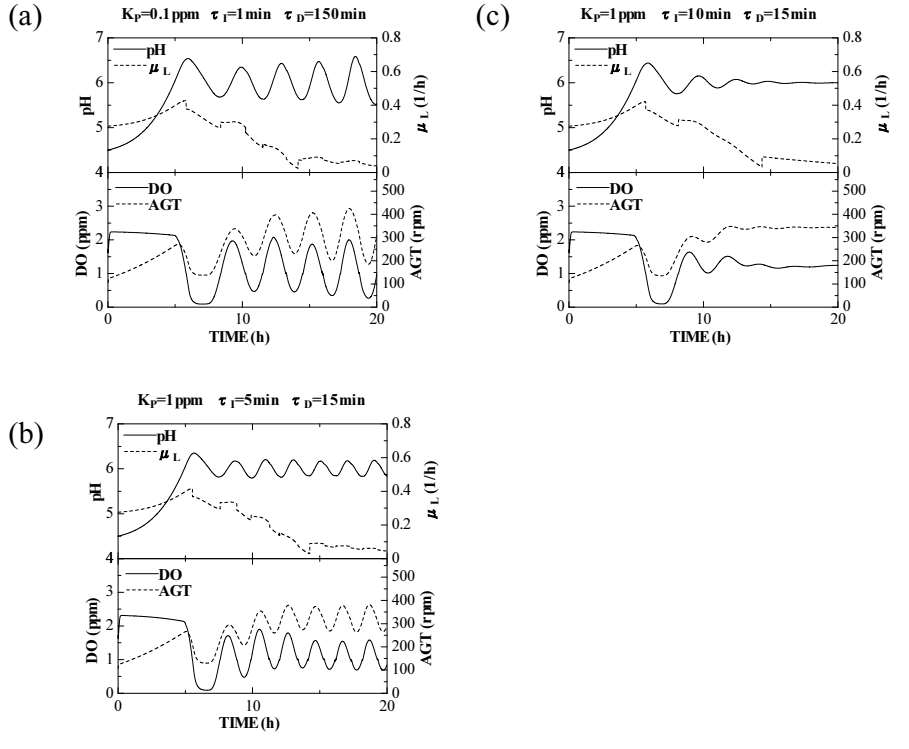

Fig. 7. Simulation results of the pH cascade control. (a) Kp=0.1 ppm, Ti=1 min,Td=150 min; (b) Kp=1 ppm, Ti=5 min, Td=15 min; (c) Kp=1 ppm, Ti=10 min, Td=15 min.

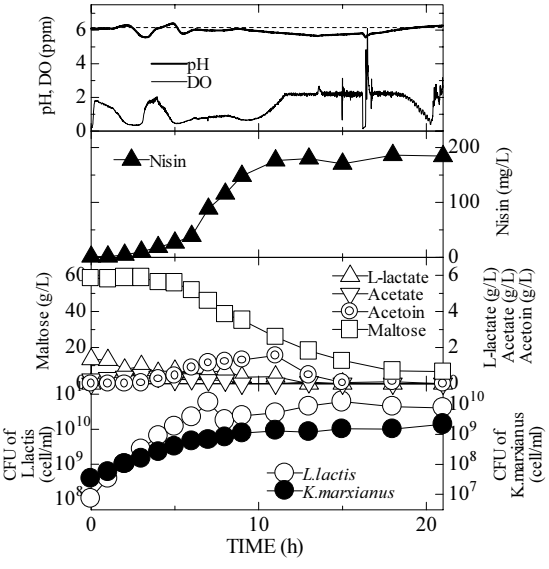

Fig. 8. Experimental result of pH control with microbial interaction

as shown in Fig.3. When the lactate concentration was expected to be decreased, the DO level should be increased, and the specific consumption rate of lactate by *K. marxianus* is enhanced.

Simulation was performed for tuning the control parameters. Parameters of the PID controller to give the set point of DO are tuned optimally, because the control performance of the PID controller was significant for entire control performance. The application of the mathematical model to optimize the performance of this control system, are shown in Figs. 7 (a), (b), and (c), respectively. A pH set point was set 6.0 in this case. The best performance of this control system to stabilize the pH was found when the parameters of the controller was set at Kp = 1 ppm, Ti = 10 min and Td = 15 min. In this condition, the PID control system was tuned so that the fluctuation was less than 0.5 units in the simulation. It was confirmed that The pH value was controlled at 6.0 during the fermentation as shown in Fig. 8. The nisin concentration finally reached at 200 mg/L, indicating the symbiotic process well worked.

8 Robustness and Stability of the Symbiotic Control Process

8.1 Experimental Evidence of the Stability of the Control Process

When inoculum size of *L. lactis* is greater than the expected value, or inoculum size of *K. marxianus* is less than the expected value, lactate is not completely assimilated by *K. marxianus* and pH decreases. In such a case, growth of

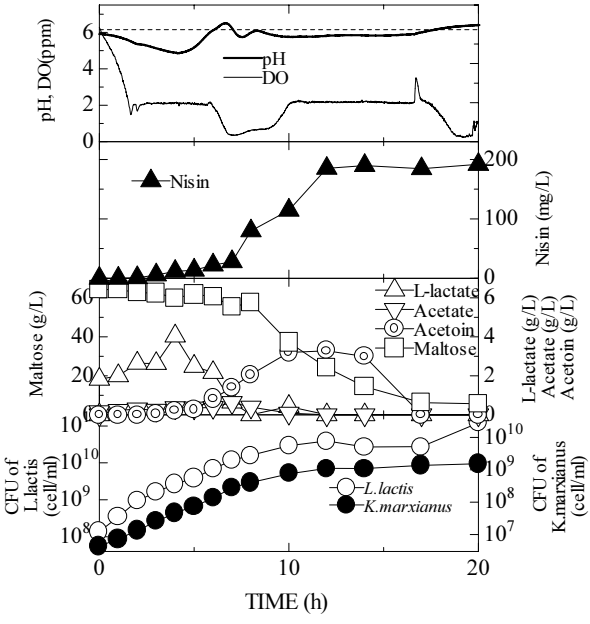

Fig. 9. Stability test for uncertainty of inoculum size

L. lactis is expected to be inhibited. Figure 9 shows the result of the stability test for uncertainty of inoculum sizes of both microorganisms. In this case, imbalance of cell of *K. marxianus* was inoculated, which is 1/10 less than that of optimal inoculum size. Due to imbalance of the cell concentrations of both microorganisms, lactate was accumulated and pH was decreased to 4.9. As a result, growth of *L. lactis* was stopped at 4.5 h. However, lactate was assimilated gradually by *K. marxianus* and both microorganisms well grew after 8h. Nisin concentration reached to 190 mg/L in this case. It was experimentally proved that this control system was robust for such uncertainty of inoculum size of microorganisms.

8.2 Symbiotic Network Analysis

It is found that a symbiotic process of a lactic acid bacterium *L.lactis* and diary yeast, *K. marxianus* shows stable behavior. The environmental conditions such as pH and DO concentration are kept constant levels. Even though the initial cell concentrations of the both cells are imbalanced, the process goes to the stable states. Interaction of both microorganisms in the symbiotic process by sharing the common metabolites and environmental condition are illustrated in Fig.10 (a). Arrows indicate enhancement of microorganisms' activities or increase in the metabolites concentrations in the environment, while stop bars indicate inhibition of microorganisms' activities or decrease in metabolites concentrations in the environmental condition. Solid and dotted lines indicate active and inactive interactions and nodes, respectively.

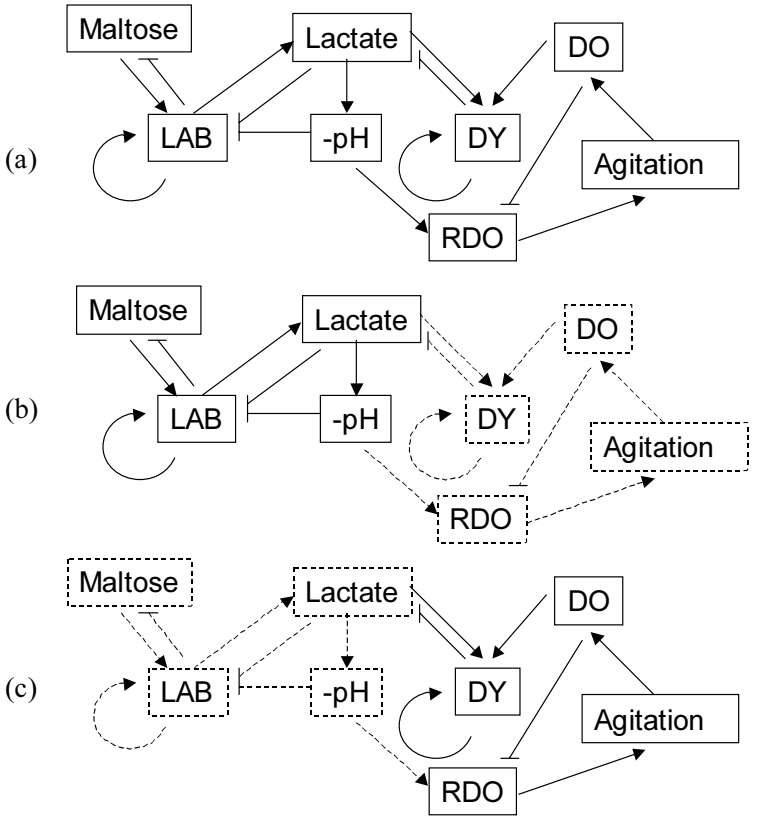

Fig. 10. Illustration of symbiotic network: behavior of symbiotic network in the cases that both microorganisms grow well (a), the *K. marxianus* concentration is much lower than that of *L. lactis* (b), and *L. lactis* concentration is much lower than that of *K. marxianus* (c), respectively. The both cases of (b) and (c) autonomously recovers to the orignial status of (a). Arrows indicate enhancement of microorganisms' activities or increase in the metabolites concentrations in the environment, while stop bars indicate inhibition of microorganisms' activities or decrease in metabolites concentrations in environmental condition. Solid and dotted lines indicate active and inactive interactions (and nodes), respectively. LAB: *L.lactis*, DY: *K. marxianus*.

When *K. marxianus* concentration is much lower than that of *L. lactis*, the action from *K. marxianus* is negligible compared with activity of *L. lactis*. The active network in this situation is illustrated in Fig.10 (b). In this case, lactate is accumulated and pH is decreased, which causes the stop of cell growth of *L. lactis* and dynamic behavior of entire network are slow down for a while. After *K. marxianus* grows as much as *L. lactis* and lactate concentration is decreased gradually, the original activity of network as shown in Fig. 10 (a) recovers autonomously. In the pure cultivation process of *L. lactis*, this autonomous recover is not possible as shown in Fig.2.

On the other hand, when *L. lactis* concentration is much lower than that of *K. marxianus*, lactate concentration goes down for sufficient growth of it K. marxianus. In this case, *K. marxianus* growth is also inactivated due to depletion of lactate, and dynamic behavior of entire network is slow down for a while as shown in shown Fig.10 (c). After *L. lactis* grows as much as *K. marxianus*, and lactate concentration is gradually increased, the original activity of network as shown in Fig. 10 (a) recovers autonomously in this case as well.

In the symbiotic processes, more than one microorganism highly interact each other as shown in the case of this study. In the case that the activity of one microorganism slows down, this issue affects on the activity of other microorganisms and activity of the entire bionetwork slows down. As a result, the entire system waits for the recovery of the activity of the microorganisms, and avoid the stuation that the only one microorganism becomes a winner. When the activity of the microorganism recovers, the entire bionetwork also recovers autonomously. This concept would be useful for creation of a autonomous recovery system in information technology.

9 Conclusions

The symbiotic process of *L. lactis* and *K. marxianus* stably worked well. The pH was well controlled by the cascade controller with the microbial interaction. This controller was robust for uncertainty of inoculum sizes of microorganisms. The indicator of the symbiotic process, nisin concentration reached 200mg/L and this value was 20 times greater than that in pure cultivation process without control of pH. The dynamic behavior and autonomous recovering process are discussed when the cell amounts of the both microorganism are imbalanced.

Acknowledgements

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